



### Complement Depletion: Use of Human C3/Cobra Venom Factor (CVF) Chimeric Proteins as Therapeutic Agents

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The complement system is part of the innate immune system, and is found in all vertebrates as well as in an increasing number of invertebrates<sup>1,2</sup>. The complement system contains over thirty proteins that are involved in three different activation cascades and one terminal pathway, as well as a number of proteins involved in regulating complement activation<sup>3</sup>. One complement component, C3, plays important roles in two of the amplification cascades, and plays the central role in the third, the alternative pathway (AP). In the AP, C3 undergoes a spontaneous conformational change that exposes multiple protein binding sites and results in the cleavage of a high-energy thioester linkage found only in C3, C4, the serum protease inhibitor  $\alpha$ -2-macroglobulin, and two recently discovered proteins, CPANMD8 and CD109<sup>4-6</sup>. The activated C3, called iC3 or C3(H<sub>2</sub>O) is able to bind another complement protein, factor B, which can be cleaved by another complement protein, factor D, to yield the AP C3/C5 convertase, iC3,Bb. This convertase can activate more C3 molecules by cleaving off the C3a peptide from the activated C3, called C3b. C3b can then bind other factor B molecules to form more C3/C5 convertases. By binding a second C3b molecule, a C3 convertase can more efficiently cleave C5 into the anaphylatoxin C5a, and the active form, C5b, which binds C6-C9 to form the membrane attack complex in the terminal complement pathway [for review, see<sup>7</sup>].

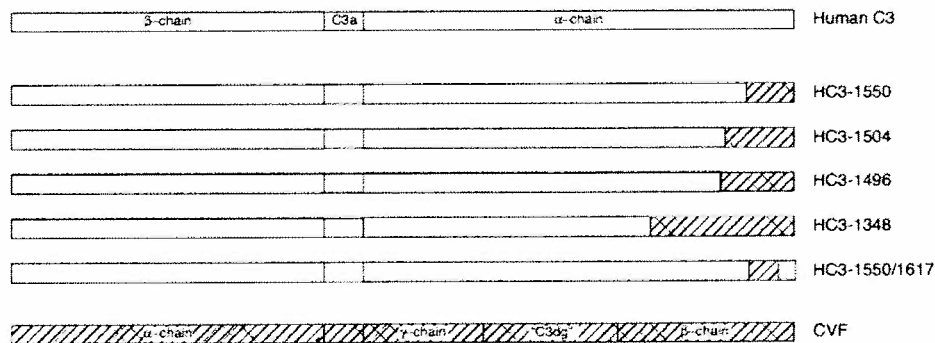
Complement activation provides the body's first line of defense against bacterial and viral disease, as complement can either target "non-self" cells for phagocytosis or cause their lysis. However, there are certain diseases, and certain situations, where complement activation is actually responsible for a majority of the disease symptoms, or resulting damage<sup>8</sup>. For example, the fact that complement activity is seen in the joint fluid of patients suffering from rheumatoid arthritis suggests the involvement of complement in this disease. Complement activation has also been implicated in other immune-mediated diseases, such as Myasthenia gravis and various forms of lupus [for review, see<sup>9</sup>]. Activation of complement has also been seen to be a cause of the tissue damage in myocardial infarctions and reperfusion injuries<sup>8</sup>. Finally, complement is a major cause of rejection of foreign tissue during xenotransplantation procedures<sup>10</sup>. For these reasons, there has been a great deal of research on substances that are able to inhibit the activation of complement<sup>11</sup>, with several compounds now in clinical trials. Examples of these therapies include antibodies to complement proteins, complement proteins that are truncated to alter their activity, and small molecules or protease inhibitors that prevent the activation of complement. One commonality among all these potential anti-complement drugs is that they inhibit the action of complement. We are now examining an alternative means

of blocking complement action by depleting the complement in an organism.

Cobra Venom Factor (CVF) is a protein found in the venom of most elapid snakes<sup>12</sup>. The protein has been extensively studied, and been shown to be an analog of complement C3. Sequencing of CVF in our laboratory has shown that it is highly homologous to C3 from many other species, with homology ranging from about 70% in mammals to >90% homology to cobra C3<sup>13</sup>. Though the mature protein has a structure similar to C3c, one of the breakdown products of C3, functionally it most resembles C3b in that it is able to bind factor B, which is then cleaved by factor D to form the CVF containing C3/C5 convertase, CVF,Bb<sup>14</sup>.

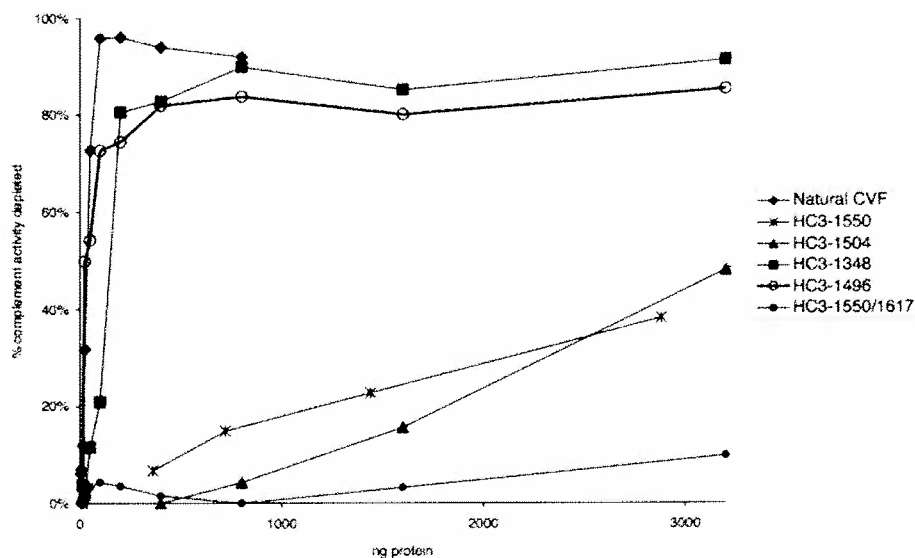
While functionally similar to C3b, CVF also shows some significant differences. Convertases formed with either C3b or CVF are inherently unstable, and will spontaneously dissociate to Bb plus C3b (or CVF). However, the CVF-containing convertase is far more stable than the C3b containing enzyme, with a  $t_{1/2}$  of approximately 7 hours<sup>14</sup> vs. 1.5 minutes<sup>15,16</sup>. The CVF-containing enzyme is also not subject to regulation and cleavage by factors H and I<sup>17-19</sup>. In addition, the CVF-containing convertase is able to cleave C5 on its own, while the C3b-containing enzyme needs to have a second C3b molecule covalently bound<sup>20</sup>. Finally, lacking a thioester, the CVF-containing enzyme remains in the fluid phase, while the C3b-containing convertase acts at the cell surface. The fact that CVF is able to form a relatively stable convertase that is resistant to the action of factors H and I means that the addition of CVF to serum results in complement depletion. Indeed, injection of CVF into laboratory animals causes complement depletion of the animal [see<sup>12</sup> and references therein]. Complement depletion by treatment with CVF has been shown to be useful for the treatment of many complement-mediated diseases in animal models [for review, see<sup>11</sup>]. In addition, it has been shown that CVF-antibody conjugates can be used for complement-mediated destruction of tumor cells<sup>21,22</sup>. However, using CVF for complement depletion does have two major drawbacks. Since complement is the first line of defense against infection, complete complement depletion could leave the patient susceptible to bacterial or viral infection. Secondly, CVF is extremely immunogenic. CVF contains  $\alpha$ -galactosyl residues at the end of the carbohydrate chains which are not found in mammalian proteins, and are highly immunogenic in themselves<sup>23</sup>. Secondly, CVF is only about 50% identical (70% similar) to mammalian C3 proteins, meaning the structure itself would be immunogenic.

In our laboratory, we are studying the structure/function relationships in complement C3 and CVF by using site-directed mutagenesis to either replace portions of human C3 with homologous CVF



**Figure 1.** — Map of portions of human C3 replaced with CVF sequences.

The figure shows the portions of human C3 (open) replaced with cobra venom factor (hatched) sequences. C3a is the anaphylatoxin portion of C3, which is removed when C3 is converted to the active form, called C3b. C3dg is the part of the mature C3 protein removed in the proteolytic conversion of C3b to C3c.



**Figure 2.** Complement depletion of human serum caused by addition of hybrid proteins.

The amounts of hybrid proteins shown were added to 10  $\mu$ l of human serum and incubated at 37  $^{\circ}$ C for three hours. Afterwards, the reaction was diluted sixteen-fold, and 10  $\mu$ l of the diluted reaction added to 30  $\mu$ l of sensitized sheep erythrocytes ( $5 \times 10^8$  cells/ml), and incubated for 30 minutes at 37  $^{\circ}$ C. Reactions were stopped by the addition of 1 ml ice-cold buffer, erythrocytes pelleted by centrifugation, and released hemoglobin measured photometrically. The graph shows the relative amount of complement activity depleted from the serum.

sequences, or to replace portions of CVF with homologous cobra C3 sequences. In doing so, we have prepared a number of human C3/CVF hybrid proteins with novel properties. With one exception, all the hybrid proteins described contain substitutions of CVF sequences at the very C-terminus of the  $\alpha$ -chain of human C3, and contain CVF substitutions of varying sizes from approximately 110 amino acid residues to 315 residues, comprising between 6.6% and 18.6% of the protein. These hybrid proteins are named HC3-1550, HC3-1504, HC3-1496, and HC3-1348, where the number is the position of the beginning of the CVF substitution in the human C3 sequence. In the one exception, HC3-1550/1617, human C3 sequences replace the C-terminal 46 amino acids of HC3-1550. Figure 1 shows a representation of the proteins used in this study.

All of these hybrid proteins show varying amounts of CVF-like behavior, in that they are able to bind factor B to form a C3/C5 convertase that is inherently more stable than the C3b-containing convertase. The convertase formed by the hybrid proteins is, in all cases, able to cleave C3, though not C5, and, surprisingly, all but HC3-1550/1617 are able to deplete complement in human serum, though less efficiently than CVF. The relative efficiencies of the hybrid proteins vary between 0.3 and 20%. Two proteins (HC3-1348 and HC3-1496) are actually able to form convertases that are more active in cleaving C3 than the CVF-containing convertase. These data are summarized in Figure 2.

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These hybrid human C3/CVF proteins show great promise for use in complement depletion. We are continuing to examine the properties of these and other human C3/CVF hybrid proteins in which substitutions are made either at the very C-terminus or in other portions of the protein. Our long-term goal is to create novel human C3-like proteins with CVF-like properties, and to define portions of C3 that are required for its function.

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